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(57) Abstract

The present invention relates to antibodies, particularly to antibodies for use in cancer therapy and diagnosis, the antibodies having substantially the same binding specificity as EXT1-7C12 monoclonal antibody. This antibody is obtainable from a hybridoma cell line deposited at BCCM under reference LMBP1360CB. The antibody is shown to bind to a variety of carcinomas with a high intensity and degree of selectivity as demonstrated by immunohistochemistry analysis and cell binding assays. Compared to a panel of other monoclonal antibodies, the EXT1-7C12 antibody demonstrates a greater selectivity in binding to tumour cells, binding especially well to tumours of the ovary. The invention also includes antibodies having substantially the same binding specificity as monoclonal antibody EXT1-7C12, and mutants, derivatives, functional equivalents or fragments of the antibody. Applications of the antibodies include the therapy or diagnosis of cancer, e.g. in targeting cytotoxic compounds to cancer.

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ANTIBODIES AND THEIR USE IN CANCER THERAPY AND DIAGNOSIS

Field of the Invention

The present invention relates to the area of cancer therapy and diagnosis, and in particular to antibodies, compositions comprising the antibodies and the use of the antibodies in cancer therapy and diagnosis.

Background of the Invention

Monoclonal antibodies have a great potential in cancer therapy as they can bind to tumour antigens with great selectivity and guide cytotoxic activities to the cancerous cells. Thus, antibodies conjugated with cytotoxic compounds such as radioisotopes, drugs or toxins can be targeted to the tumour cells and inflict tissue damage on the tumours with varying degrees of selectivity (Goodman et al, 1993). Also naked antibodies can be used utilising natural defence mechanisms such as antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) against the tumour cells (Mujoo et al, 1987).

The degree of success in therapies using the antibodies depends on many factors, but an important factor is the restricted expression of the antigen recognised by the antibody to the tumour cells. Likewise, the extent of cross-reactivity of the antibody with other molecular structures and normal tissues is highly important. Thus, the ideal antibody would only bind to antigens expressed on tumour cells and not to any normal cells. Since most, if not all, tumour associated antigens are expressed both on normal as well as tumour cells (Sakamoto et al, 1986), the problem in the art has been to develop antibodies towards antigens expressed to a much higher degree on tumour cells than on normal cells.

The ability of any given monoclonal antibody to discriminate between tumour and normal tissue can to a large extent be determined by *in vitro* tests or animal model tests. Examination of the ability of the antibody to bind to tumour

cells with selectivity can be performed with, for example, immuno-histochemistry, FACS analysis and other cell binding tests (Mittoti et al, 1987). Also the ability of the antibody to mediate cytotoxic effects can be studied *in vitro* (Cheresh et al, 1985). Additional data concerning the suitability of the antibody to be used as a therapeutic or diagnostic drug can be gathered from animal model studies (Schreiber et al, 1992; Fujita et al, 1992; Isaacs et al, 1994).

However, in many cases, only studies performed on patients will truly reveal the pertinent characteristics of the antibody. A large panel of monoclonal antibodies have been, and are presently, in various stages of clinical trials (Labus et al, 1992; Kalofonus et al, 1989; Friedman et al, 1993; Goodman et al, 1993), but many of these monoclonal antibodies have failed to demonstrate significant clinical efficacy in extended trials. However, a recent report where a murine monoclonal antibody, 17-1A, which is directed on an epitope on colo-rectal carcinomas, shows that this antibody is highly effective in prolonging the survival of patients over a five year period (Reitmuller et al, 1994).

Summary of the Invention

The applicants have developed a murine monoclonal antibody (EXT1-7C12) after PEG-induced fusion of murine splenocytes, immunised with human tumour cell lines, to HAT-sensitive SP2/0 myeloma cells. The antibody binds to a variety of with a high intensity and degree of selectivity as demonstrated by immunohistochemistry analysis and cell binding assays. The antigen to which the antibody binds has not yet been fully identified, but is most probably a comparingly large membrane associated glyco-protein. The antigen is shed from the tumour cells and can be found in patients blood or in spent culture medium from antigen expressing cells. Compared to an array of other monoclonal antibodies EXT1-7C12 demonstrates a more selective binding to tumour cells, binding especially

well to tumours of the ovary. The antibody has been demonstrated to target to human tumours transplanted into immunodeficient mice. Thus, the EXT1-7C12 monoclonal antibody shows unique features in binding activity and selectivity of human tumours and might prove useful for diagnosis and therapy of human tumours.

Accordingly, in one aspect the present invention provides antibodies having substantially the same binding specificity as monoclonal antibody EXT1-7C12 produced by the hybridoma cell line EXT1-7C12-B7-AA4-7A2-2C2 deposited at BCCM LMBP Culture Collection, Laboratory of Molecular Biology, University of Gent, K.L.Ledeganckstraat 35; B-9000 Gent, Belgium, the 14 March 1995, under reference LMBP1360CB.

In a further aspect, the present invention provides antibodies as obtainable from the hybridoma cell line deposited at BCCM under reference LMBP1360CB.

In a further aspect, the present invention provides a mutant, derivative, functional equivalent or fragment of antibody EXT1-7C12 cr an antibody having the binding specificity of the EXT1-7C12 antibody. Various methods for producing such modified antibodies are discussed at greater length below.

In a further aspect, the present invention provides the hybridoma cell line deposited at BCCM under reference LMBP1360CB.

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more of the above antibodies, in combination with a pharmaceutically acceptable carrier.

In a further aspect, the present invention provides an antibody as described above for use in methods of medical treatment or diagnosis. In particular, the present invention

use of one or more of the above antibodies in the preparation of medicaments for use in cancer therapy and diagnosis, and more especially in the therapy or diagnosis of ovarian cancer.

In a further aspect, the present invention provides a method of determining the concentration or presence of cancer cells using the above antibodies. One method involves associating the antibodies with a label, exposing cells in a test sample to the antibodies and determining the extent of binding by detecting the label.

The specificity of the antibodies of the present invention makes them particularly useful in targeting therapies to cancer cells. As discussed above, these targeting therapies are well known in the art and include so called ADEPT or VDEPT techniques in which an active agent is administered as a precursor, for conversion to an active form by an activating agent targeted to or produced in the cells to be treated. Thus, the activating agent can be targeted to the cells by conjugation to antibodies of the present invention.

The antigen to which the EXT1-7C12 antibody binds has not yet been fully identified but is most probably a membrane integral glycoprotein. The unique binding profile of the antibody also indicates that it has not been described earlier. Compared to an array of other monoclonal antibodies EXT1-7C12 demonstrates a more selective binding to tumour cells. In particular, tumours of the ovary are intensely stained.

The antibody has been demonstrated to target to human tumours transplanted into immunodeficient mice. The EXT1-7C12 monoclonal antibody, thus, shows unique features in binding activity and selectivity of human tumours, useful for the diagnosis and therapy of human tumours.

The EXT1-7C12 antibodies have a number of *in vivo* therapeutic applications. In addition to being used alone to target tumour cells, the antibodies may be used in conjunction with an appropriate therapeutic agent to treat human cancer. For example, the antibodies linked to therapeutic drug, such as cytostatica, cytokine or a radioisotope, for example ^{188}Rh or ^{90}Y or toxin, such as abrin or ricin for delivery of the therapeutic or toxic agent tumour cells according to well-known techniques.

The EXT1-7C12 may be administered *in vivo* or *ex vivo*, alone or in combination with one or more other antibodies.

The invention includes pharmaceutical compositions comprising a therapeutical effective amount of EXT1-7C12, either unmodified or a mutant, derivative, functional equivalent or fragment thereof, it may be conjugated or a part of an antibody cocktail for the purpose of treating cancer.

The antibody composition of the invention can be administered using conventional modes including intravenous, intraperitoneal, oral, intralymphatic or directly into the tumour. Preferably the antibody is administered parenterally.

The dosage forms may include liquid solutions, or suspensions, tablets, microcapsules, liposomes and injectable solutions. The precise dosage form will be dependent upon the mode of administration and the therapeutic application, and can be readily determined by the person of ordinary skill in the art.

The pharmaceutical compositions preferably include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate and salts of electrolytes.

Modified Antibodies

The antibodies described above can be altered in a variety of ways using recombinant DNA technology coupled with advances in the field of monoclonal antibody and protein engineering. This has enabled access to a large selection of antibodies and antibody fragments with different properties and structures to natural antibodies.

As discussed above, the production of monoclonal antibodies is well established in the art. Monoclonal antibodies can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma producing a monoclonal antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with the target, or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest (or a fragment thereof).

Antibodies may be modified in a number of ways. Indeed the

term "antibody" should be construed as covering any specific binding substance having a binding domain with the required specificity. Thus this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (eg by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the

first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. 4, 446-449 (1993)), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker et al, Embo Journal, 10, 3655-3659, (1991).

Bispecific diabodies, as opposed to bispecific whole antibodies, are also particularly useful because they can be readily constructed and expressed in *E.coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

Previously, bispecific antibodies incorporating a specificity for the T-cell co-receptor CD3 have been shown to inhibit tumour growth (Titus, J. A. et al, J. Immunol. 138, 4018-4022 (1987)) and to cure lymphoma (Brissinck J. et al, J. Immunol. 174, 4019-4026 (1991)).

It may be desirable to "humanise" non-human (eg murine) antibodies to provide antibodies having the antigen binding

properties of the non-human antibody, while minimising the immunogenic response of the antibodies, eg when they are used in human therapy. Thus, humanised antibodies comprise framework regions derived from human immunoglobulins (acceptor antibody) in which residues from one or more complementary determining regions (CDR's) are replaced by residues from CDR's of a non-human species (donor antibody) such as mouse, rat or rabbit antibody having the desired properties, eg specificity, affinity or capacity. Humanised antibodies may be prepared according to well known techniques in the art and are illustrated herein by the references Jones P.T., Dear P.H., Foote J., Neuberger M.S. and Winter G. "Preparing the complementary-determining regions in a human antibody with those from a mouse", Nature, 321: 522-525 (1986) and Verhoeyen M.E., Saunders J.A.,Price M.R., Marugg J.D., Briggs S., Broderick E.L., Eida S.J., Mooren A.T.A. and Badley R.A. "Construction of a Reshaped HMFG1 Antibody and Comparison of its Fine Specificity with That of the Parent Mouse Antibody", Immunology 1993, vol 78 pages 364-370. Some of the framework residues of the human antibody may also be replaced by corresponding non-human residues, or by residues not present in either donor or acceptor antibodies. These modifications are made to the further refine and optimise the properties of the antibody.

The present invention will now be described by way of example with reference to the following drawings.

Brief Description of the Drawings

Figure 1 shows the cloning steps leading of the EXT1-7C12 antibody leading to the master working cell bank;

Figure 2 shows the staining of the Ovarian carcinoma cell line NIH:OVCAR-3 with EXT1-7C12

Figure 3 shows the binding of radiolabelled EXT1-7C12 to NIH:OVCAR-3 cells

Figure 4 shows the uptake of radiolabelled EXT1-7C12 in different tissue types;

Figure 5 shows the binding of EXT1-7C12 to purified antigen;

Figure 6 shows a western blott of a blott from a SDS-PAGE of shed antigen detected with EXT1-7C12 antibody;

Figure 7 shows the EXT1-7C12 antigen to be different from the MUC-2, MUC-3, MUC-4 and MUC-5 antigens;

Figure 8 shows the ELISA detection of EXT1-7C12 in the circulation of patients with ovarian cancer; and,

Figure 9 shows the lack of correlation between the ovarian cancer marker CA125 and EXT1-7C12.

Detailed Description

Production and characterisation of the hybridoma cell line and the EXT1-7C12 monoclonal antibody

Immune parental cell

Immune spleenocytes were obtained from Balb/c mice (Charles River UK. Ltd, Margate Kent). The mice which were negative for a number of pathogenic virus and bacteria were injected intraperitoneally (i.p.) with a mixture of proteins extracted from the human ovarian tumour cell line, NIH:OVCAR-3(Hamilton et al, 1983) in accordance with the following schedule.

Date	Amount of prep.	Adjuvant
920305	0.25 ml subcutaneously	0.25 ml FCA
920325	0.25 ml subcutaneously	0.25 ml FCA
920410	0.25 ml intra peritoneal	None
920602	0.25 ml intra peritoneal	None

FCA = Freunds Complete Adjuvant

NIH:OVCAR-3 is an ovarian carcinoma cell line. The cells were cultivated without addition of antibiotics in R10 medium, RPMI 1640 (Gibco, No 041-08170) with addition of L-glutamine (Gibco, No 043-05030), "non essential aminoacids" (Gibco, No 043-01140) and foetal calf serum (FCS, Gibco, No 011-06290). The cells were regularly checked for contaminating mycoplasma with the fluorescent dye Hoechst 33258 (Chen, 1972), an ultramicrochemical test (Uitendaal

et al., 1978) and with a filter hybridization technique using ^{32}P labelled restriction fragment H900 as a radioactive DNA-probe specific for mycoplasma (SVA) and were always found negative. The cells were kept in serial cultures and removed from the tissue culture flasks using a trypsin EDTA-solution (Gibco No 45300-019). The cells were washed in PBS before being inoculated into the mice. A solid NIH:OVCAR-3 ovarian carcinoma was established subcutanously in a SCID mouse (tested and found negative for virus and pathogens). The animal was transplanted with $1x10^6$ viable NIH:OVCAR-3 cells and the animal was killed when the tumour reached a size of 1 x 1 cm. The tumour was frozen and later thawed, minced with scissors and mixed with destilled water to make a final 20% w/v of tissue in water. The mixture was then run in a Omnimixer at speed setting 6 for 1 min. The preparation was admixed to an equal amount of 1.25 M perchloric acid and mixed with a magnetic stirrer at room temperature for 30 min. Insoluble material was finally spun down for 30 min at 27 000 g, and the supernatant was dialysed against water and PBS (PBS = 8 mM Na_2HPO_4, 2 mM KH_2PO_4,137 mM NaCl, 3mM KCl, pH 7.3)to create the tumour cell extract used for immunization.

A mouse was injected four times with 250 ul doses of the tumour celline extract. The last injection was a booster dose given three days before fusion.

Three days after booster inoculation of the mouse 120 $x10^6$ viable spleen cells were harvested and fused with $60x10^6$ SP2/0-Ag-14 myeloma cells using polyethylenglycol 1540 (Polyscience Inc, PA, No 1102). The SP2/0 myeloma cells were taken from a master working cell bank and the cells were kept in exponential growth phase and cultivated for less than 1 month prior to the fusion. The fusion mixture was distributed into Costar 96-well tissue culture plates at a cell density of 129 x $10^3/100\mu l$/well. Hybridomas were selected in DMEM-HAT medium (Gibco, No 041-01965 and No

043-01060) supplied with HECS (Human Endothelial Cell Supernatant, Costar, No C6110) used according to the manufacturers recommendation and with 10% foetal calf serum (Gibco, No 011-6290). The wells were supplied with fresh medium each week until supernatants from wells with growing hybrids were screened for binding activity against tumour cell lines utilising cell-ELISA techniques (se below).

Five different wells were classified as positive and were then cloned through limiting dilution. One of the wells was cloned four times seeding 1 cells/well for the first cloning and 0.1 cells/well for the last cloning step as illustrated in figure 1. The various steps were characterised by the following:

Fusion 920605
ELISA 920623
3 per cent of growing hybrids positive in cell-ELISA
Cloning 1 920703 1 cell/well
ELISA 920714
100 per cent of growing hybrids positive in cell-ELISA
Cloning 2 920728 0.2 cell/well
ELISA 920807
100 per cent of growing hybrids positive in cell-ELISA
Cloning 3 920904 0.1 cell/well
ELISA 920917
100 per cent of growing hybrids positive in cell-ELISA
Cloning 4 930930 0.1 cell/well
ELISA 931010
100 per cent of growing hybrids positive in cell-ELISA

This procedure resulted in the monoclonal hybridoma line EXT1-7C12-B7-AA4-7A2-2C2 and this cell line was expanded to Master Cell Bank (25 amp) and further to a Manufacturing working Cell Bank (50 amp) (figure 1). The monoclonality of the hybridoma cell line was ascertained by the fact that during the last two cloning steps all wells with growing hybridoma cells also produced antibodies binding to the

antigen.

Further analysis including PAGE, isoelectric focusing, FPLC and determination of isotype have all confirmed this conclusion. The cloning procedure was performed in HECS-supplemented medium without addition of feeder cells.

The EXT1-7C12-B7-AA4-7A2-2C2 monoclonal hybridoma cell line produces a monoclonal antibody called EXT1-7C12 which is a murine IgG_1 kappa antibody as determined by immunodiffusion and ELISA assays using isotype-specific (ICN Biomedicals Inc, USA) and light chain specific (Southern Biotechnology Associated Inc, USA) antibodies respectively. The hybridoma was found to produce 5.2 μg antibody/ml/10^6 cells/24h under exponential growth in a tissue culture flask. No production of immunoglobulin originating from the myeloma cell line could be found, and analysis of the sequence of parental mRNA coding for Ig-chains revealed the inclusion of a stop codons and modifications in the Ig-genes obliterating the translation of mRNA into protein.

Activity of the antibody

Binding to tumour cell lines as analysed with ELISA

Monolayers of tumour cells in 96 well tissue culture plates were dried at 37°C over night and fixed with 0.025% glutaraldehyde in PBS for 10 min. After fixation the plates were washed in PBS and residual glutaraldehyde was inactivated with 50% FCS in 0.1M glycine, 50 mM TRIS-HCl buffer pH 7.8. The wells were washed with 0.01 M sodium phosphate buffer pH 7.3, containing 0.15 M sodium chloride and 0.05% Tween 20. Culture supernatants to be tested for presence of anti-tumour antibodies were serially diluted, added to the wells containing tumour cells and incubated for one hour at 37°C. Bound antibody was then detected with horseradish peroxidase (HRPO) conjugated to a rabbit anti-murine Ig (P260, Dako A/S, Glostrup, Denmark) for one

hour. After additional washing steps 200 µl of developing solution (3.7 mM ortho-phenyldiamine in 0.05 M citric acid, pH 5.0) was added to each well. The reaction was stopped after 10 min by adding 50 µl of 1M H_2SO_4 to each well, and the absorbance was read at 492 nm in a Multiscan MCC/340. Absorbance three times above the media background was regarded as positive (+). A summary of the results is presented in Table 1.

Binding to tumour cell lines as analysed by flow cytometry

The tumour cell lines were tested for reactivity with hybridoma supernatants using an indirect immunofluoresence assay. Adherent cell lines were treated with trypsin and washed in PBS containing 1% bovine serum albumin (PBS-BSA) while non adherent cells were incubated directly with antibody containing culture supernatant. The cells (0.5-1x10^6) were incubated with 100 µl diluted hybridoma cell culture supernatant for 45 min on ice, washed as above, and reincubated for 45 min on ice with a FITC conjugated goat anti-murine Ig (Dako A/S). The cells were then washed and analysed on a FACStar plus flow cytometer (Becton Dickinson, San José, CA) as illustrated in Figure 2. The white signal represents the EXT1-7C12 stained NIHOVCAR-3 cells and the black signal represents the background signal from cells stained with the negative control. A summary of the results is presented in Table 2. A variety of tumour cell lines with hematopoietic origin were tested, but none of them were stained with the EXT1-7C12 antibody.

Differences were found between results obtained from the ELISA-tests and flow cytometric analysis as, for example PC-3 was stained when analysed in ELISA, but was found negative in flow cytomeric tests. Possibly the trypsin used in the flow cytometry tests reduced the amount of antigen on the cell surface.

Immunohistochemistry

Tissue specimens used for immunostaining were removed during surgery or autopsy and snap frozen in isopentane within one hour after removal. The tissues were cut into 5μm thick sections using a cryotome. The sections were dried over night at room temperature and fixed in acetone at -20°C for 10 min. After fixation, the sections were soaked for 30 min at +20°C in TRIS buffer containing 10% FCS and then incubated at room temperature for 1 hour with antibody containing supernatants, diluted to give a final Ig-concentration of 5-10 μg/ml. The sections were washed 3 times and biotin labelled goat anti-murine Ig was added. After incubation for 30 min a horeseradish peroxidase avidine biotin complex (VECTASTAIN ABC-kit, Vector Laboratories Inc., Burlingame, CA) was added and incubated for 30 min. The sections were washed and supplied with a solution containing diaminobenzidine (DAB) to visualize bound antibody. After a final washing step, the sections were dried and mounted in mounting media (PERTEX, Histolab, Sweden) before examined in a microscope. Tumours were considered positive if more than 10% of the tumour cells were clearly stained. In many tumours, all neoplastic cells were found to express the antigen. The staining was associated with the plasma membrane of tumour cells and gave an overall impression of a mucin type of staining. A summary of the staining results is shown in Table 3. The EXT1-7C12 antibody bound a large portion of human ovarian carcinomas, but also to breast and lung carcinomas. The EXT1-7C12 also bound to some normal tissue, although to a low frequency and with a low intensity.

The EXT1-7C12 also bound to formalin fixed and paraffin embedded tissue. When a number of routine pathological ovarian tumour specimens were tested for EXT1-7C12 binding it was found that most of the tumours were positively stained (Table 4).

The results from this investigation revealed that the EXT1-

7C12 antibody bound a high frequency of ovarian carcinomas. It is also clear from table 4 that EXT1-7C12 binds the majority of the serous cystadenomas, the most frequent type of ovarian tumour. Since it is well known that most, if not all, monoclonal antibodies to tumour associated antigens also bind to normal tissue to varying degrees, the staining pattern of the EXT1-7C12 antibody was compared to that of four other murine monoclonal antibodies already used in clinical trials for immunolocalization and therapy of tumours (Table 5).

The 17-1A (ReitmŸller et al, 1994) and KS 1/4 (Labus et al, 1992) monoclonal antibodies which probably recognize different epitopes on a tumour associated antigen present on many different carcinomas (Strnad et al, 1989) together with HMFG-1 (Epenetos et al, 1987, Kalofonos et al, 1989, Hammersmith Oncology group 1984), which was raised against a human mucin (Taylor-Papadimitriou et al, 1981) present in many different types of adeno carcinomas (Girling et al, 1989) and the B72.3 antibody (Esteban et al, 1987) recognizing a sialoyl containing carbohydrate antigen enriched on many different forms of tumours (Ohuchi et al, 1986; Xu et al, 1990; Cordoncardo et al, 1992; Bryne et al, 1990; Inoue et al, 1991; Johnston et al, 1986) were evaluated. A nonbinding IgG_1 antibody and cell culture medium were used as controls.

When compared with the four monoclonal antibodies described above the EXT1-7C12 was found to be as broad in its tumour interaction as they were, but had much less cross reactivity with normal tissue (Table 5).

The 17-1A and KS1/4 antibodies showed a more homogeneous binding to the ovarian tumours tested. However, their binding to normal tissue was also very pronounced. Besides binding to epithelial cells in kidney, colon, stomach, pancreas, oesophagus and breast the KS1/4 also bound to

peripheral nerve and to endothelium in small blood vessels in brain and placenta. In the liver, both the bile collecting ducts and some arteries were stained. The intensity of the staining was as strong in normal tissue as in tumour tissue. 17-1A showed a similar pattern, but stained weaker than KS1/4. Staining with both HMFG-1 and B72.3 was associated with mucin producing tissue and stained the apical parts of tumour as well as normal cells. Both antibodies also stained kidney tubule, normal epithelia in ducts of the breast and the ascini of pancreas. HMFG-1 stained all ovarian carcinomas but the intensity of the staining was similar in normal colon and tumour tissue. In our hands, the HMFG-1 antibody stained all normal striated muscle tested with a low intensity. The B72.3 antibody also stains cell nucleus and some normal blood vessels in the brain, this staining was weak but reproducible and found in most brain tissue tested. A staining of blood vessels in the brain was also observed with KS1/4.

In conclusion, the EXT1-7C12 showed both less frequent and less intense cross reactivity with normal tissue as compared to the other monclonal antibodies.

It is foreseeable that the EXT1-7C12 antibody, if given to patients, could reach quite high concentrations in plasma and in tissue. To study whether such high concentrations could lead to unspecific binding to normal tissue various concentrations of the EXT1-7C12 antibody, and for comparison the KS1/4 antibody, were titrated from 100 to 0.0001 μg/ml on human tissue sections and evaluated by immunohistochemistry (Table 6). Estimating the plasma volume in a patient to be around 5 liters the highest dose used here in IHC (100 μg/ml) would correspond to a dose given to patients in excess of 500 mg.

The binding of EXT1-7C12 to tumour was more intense than

that of the KS1/4 antibody. The EXT1-7C12 still gave a clearly detectable staining on transplanted NIH:OVCAR-3 even when incubated at such low concentration as 0.01 mg while the KS1/4 antibody was found negative at concentrations below 0.3 mg/ml. The staining of normal tissue was however less intense. The EXT1-7C12 antibody only faintly stained normal tissue and only when used at the highest concentration.

This indicates that the antigen recognised by the EXT1-7C12 antibody was absent or only present in a very low concentration in normal tissue. The results also suggest that the EXT1-7C12 antibody, when administered to patients in doses less than 5 mg, will not bind unspecifically to tissues not expressing the antigen.

In vivo activity of the EXT1-7C12 antibody

Ability of the EXT1-7C12 antibody to localise to human tumours transplanted to immunodeficient SCID mice

Localisation of unlabelled antibody

SCID mice which had been tested and found to have a serum-concentration of murine antibodies less than 1 mg/ml were used in these studies. The mice were inoculated subcutaneously with 0.1 ml cell suspensions containing 1 - 2 x 10^6 viable tumour cells in each shoulder. Each animal was supplied with one EXT1-7C12 antigen negative (M24 met) and one EXT1-7C12 antigen positive tumour (NIH:OVCAR-3). The tumours were allowed to grow to the size of approximately 8x8 mm when the animals were injected i.p. with 250 mg of purified EXT1-7C12 antibody dissolved in sterile PBS. The animals were sacrificed 1, 2 or 3 days after injection of antibodies and both tumours as well as liver and kidney were frozen and analysed in immunohistochemistry for the presence of antibody.

The experiment showed that antibody localised well and immunoglobulin could be found in the antigen expressing NIH:OVCAR-3 tumour from day 1 onwards, whereas the M24 met tumour did not contain any detectable antibody. Less but still significant amounts of antibody were also found in the liver and to some extent in the kidneys. The presence of minor amounts of antibody in liver and kidney might be explained by the fact that levels of antibody was found in the circulation of the animals throughout the experimental period.

Localisation of radiolabelled antibody to transplanted tumours

Purified EXT1-7C12 antibody were labelled with ^{125}I using the ATE method (Schuster et al, 1991). In brief, 50 µl 1% acetic acid in methanol, 25 µl N-succininimidyl-3-(tris-n-butylstannyl)benzoate (ATE) 1mg/ml, 12 µl of N-

chlorosuccinimide 5mg/ml and 30 µl Na I^{125} (114 MBq, Amersham, IMS 30) was mixed in a glas tube and incubated for 15 min. The reaction was terminated by the addition of 10µl NaS_2O_3 5mg/ml and stirred for another 5 min. The mixture was evaporated to dryness under nitrogen.

72 µl of EXT1-7C12 (7 mg/ml) in 0.2 M borate buffer, pH 8,7 were added to the tube, mixed and incubated at room temperature for 10 min. Then 300 ml of 20 mg/ml glycine in borate buffer pH 8,7 was used to terminate the labelling reaction under an additional 5 min. The labelling efficiency were checked with TLC (thin layer chromatography). Labelling efficiency (the amount of added iodine adhering to the protein) was estimated to 56% and the specific activity of the antibody was determined to be 63MBq/500 mg.

The radiolabelled antibody was then separated from free iodine through gelfiltration on a Pharmacia PD-10 columns equilibrated with 0.1 M Phosphate buffer, pH 7.2 and pretreated with 1% solution of BSA.

To evaluate the affinity constant of the EXT1-7C12 antibody labelled with ^{125}I was incubated with NIH:OVCAR-3 cells. Briefly, $1x10^6$ NIH:OVCAR-3 cells and 100 ml of different amounts of EXT1-7C12 antibody were mixed in tubes and incubated on ice for 60 min.

The mixture was then centrifuged through 200 ml of 40% of Ficoll to separate bound antibody from free. Radioactivity in supernatant (free) and cell pellet (bound) was then determined in a gamma counter (Table 7).

Samples of 50 ml of the supernatants were removed and analysed in a gamma counter for determination of the amount of unbound antibody. The tube were then frozen, the cell pellet containg the bound antibody were cut off and

analysed in a gamma counter giving the amount of bound radioactivity (Table 7).

The data presented in table 7 was then analysed in a Scatchard plot (Figure 3). When the amount of cell bound antibody (B) was plotted against cell bound antibody/free antibody (B/F) the affinity constant could be determined to be 0.9×10^9 M^{-1} and the number of epitopes per cell on NIH:OVCAR-3 cells to be 230 000 antibody epitopes/cell (Figure 3).

The ability of radiolabelled EXT1-7C12 antibody to target selectively to antigen-expressing tumours NIH:OVCAR-3 and not to M24 met tumours not expressing the antigen was studied in SCID mice. The tumours were established on the hinds of SCID mice as described above. The tumours were allowed to reach an average size of 8 x 8 mm. A total amount of 10 mg of EXT1-7C12 antibody with a total activity of 1.3 MBq was injected in the tail vein of each mouse. The animals were observed with a gamma camera 6, 24, 48, 72, 96 and 124 hours after injection of the antibody. Localisation of antibody to the antigen positive tumour NIH:OVCAR-3 on the right side of the animal could be observed whereas no accumulation of radioactivity was found to the antigen negative tumour (Figure 4). However, a large fraction of the radiolabel was found in the abdomen and thorax of the mice. Since the thyroid gland had not been blocked by an excess of free iodine, a successfully increasing amount of radioactivity was also found to the area of the thyroid gland. A time dependent increase in the percentage of total radioactivity localised to the antigen expressing tumour was also indicated. To obtain more accurate measurements of the distribution of the radiolabel five animals were sacrificed on each time point and different organs were removed and analysed for radioactivity in a gamma counter. The percentage of injected dose/g was calculated and the data are presented

in figure 4a. The antibody was found to localise with preference to the antigen expressing tumours. Radioactivity was not found to accumulate in any tissue exept the NIH:OVCAR-3 tumour and the thyroid gland (figure 4b).

Characterisation of the antigen

The exact nature of the antigen and the specific epitope identified by the EXT1-7C12 antibody have not yet been fully characterised. However, several findings suggest the antigen to be a protein associated with the plasma membrane of tumour cells. Moreover, the antigen is shed and could be purified from the culture supernatant or from plasmamembrane. The surfaces of viable NIH:OVCAR-3 cells were labelled with ^{125}I using the jodogen method (Fraker et al, 1978) the cells were lysed in 0.5 % Triton X-100 and the antigen precipitated with EXT1-7C12 antibody and protein A conjugated to sepharose. The antibody precipitated a large molecule that enters into the very upper part of a 8 % SDS-PAGE gel, corresponding to a protein larger than $1x10^6$ Da in molecular weight.

EXT1-7C12 antibody conjugated to Sepharose was used for affinity purification of the antigen found in the supernatant from cultivated NIH:OVCAR-3 cells. The affinity-column was eluted with 2 M guanidinium chloride and the collected antigen was dialysed against PBS buffer and used to coat 96 well microtiter plates. In ELISA analysis, the eluate gave high intensity signal with the EXT1-7C12 antibody, while other antibodies specific for large mucin molecules and with similar reactivity in immunohistochemistry (HMFG-1, HMFG-2 and SM-3) were negative (Figure 5a). In addition, the EXT1-7C12 did not bind to a synthetic peptide containing the 20 aminoacids (TAPPAHGVTSAPDTRPAPGS) from the tandom repeat of the MUC-1 mucin recognised by the monoclonal antibodies HMFG-1, HMFG-2 and SM-3 (Figure 5b).

The affinity purified antigen was separated on a gradient 4-20% SDS-PAGE gel and blotted to a nitrocellulose paper and analysed with Western blotting technique. Increasing amounts of antigen was separated on two identical gels and blotted to nitrocellulose paper. Blot a) was incubated with EXT1-7C12 and blot b) was incubated with a control antibody. Peroxidase conjugated rabbit anti-mouse antibody was used as a secondary step and binding was revealed with chemiluminisens (Figure 6). Shed antigen was similar to plasma membrane bound antigen in its migration in SDS-PAGE (data not shown).

The epitope seems to be of peptide nature since it was insensitive to treatment with endo or exoglycanases. Treatment with neuraminidase, β-galactosidase, Endo-F/peptide-N-glycosidase F or Endo a-N-acetylgalactosaminidase did not alter the binding of the antibody when tested in ELISA (data not shown). Neither did oxidative treatment with 10 mM sodium periodate or mercaptoethanol change the binding of the antibody (Figure 5a). Treatment with proteases did however effect the binding. Overnight treatment with high concentration of thermolysin did lower the binding of the EXT1-7C12 antibody in ELISA.

According to the results with the HMFG-1, HMFG-2 and SM-3 antibodies the antigen is different from MUC-1(figure 5). In order to examine if the affinity purified antigen is associated with any other known mucine, the antigen preparation was dotted to a nitrocellulose membrane and treated with hydrofloride (HF) in order to remove all carbohydrate side chains without disrupting the apoprotein (Mort et al, 1977). The purified EXT1-7C12 antigen was diluted and placed into small bands on six different nylon membrane. The amount of antigen applied to the membrane was from the top 40 mg, 8 mg, 1.6 mg, 0.32 mg, 64

ng, 13 ng, 3 ng, 0.5 ng and 0.1 ng. Membrane 1 was left untreated, while the rest of the membranes were treated with HF to remove all carbohydrates.

Membrane 1 and 2 were incubated with EXT1-7C12 and the other membranes were incubated with different polyclonal antisera specific to MUC-2 (3), MUC-3 (4), MUC-4 (5) and MUC-5 (6). Bound antibody were detected with a secondary antibody conjugated with alkaline phosphate and the bands were visualised with NBT (Figure 7).

The antigen turned out to be negative to al of the reagenses and no specific binding could be detected in the HF treated antigen. Only the higest concentration of antigen gave a weak background staining of the antigen. The antigen was also found to lose its EXT1-7C12 epitope after HF treatment.

Since the antigen is shed from cultivated tumour cell lines, the antibodies disclosed herein can be used to detect the antigen circulating in tumour patients. To verify this, we performed an analysis using a sandwich assay consisting of EXT1-7C12 as both catching and detecting antibody. 100ml of 2 mg/ml of EXT1-7C12 antibody was coated tc the wells of a 96 well plate for 1 h in 37°C. The wells were washed with PBS 0,05% Tween 20 (0.01 M sodium phosphate buffer pH 7.3, containing 0.15 M sodium chloride and 0.05% Tween 20). Sera from tumour patients and healthy blood donors were incubated for 45 min diluted 1/5 in PBS 0.05% Tween 20. After washing with the same buffer the wells were incubated with biotin labelled EXT1-7C12. Bound antibody was then detected with horseradish peroxidase (HRPO) conjugated to streptavidin(Dako A/S, Glostrup, Denmark) for 45 min. After additional washing steps, 200 µl of developing solution (3.7 mM ortho-phenyldiamine in 0.05 M citric acid, pH 5.0) was added to each well. The reaction was stopped after 10 min by adding

50 µl of 1M H_2SO_4 to each well, and the absorbance was read at 492 nm in a Multiscan MCC/340. EXT1-7C12 units were calculated from 100 x OD (492). In figure 8, the results from patients with advanced decease are compared with the results from blood donors. The results from the tumour patients were also compared to the CA125 values (Abbot diagnostic) from the same sera (Figure 9).

Conclusions of preclinical evaluation

The EXT1-7C12 antibody differs from other antibodies recognising tumour associated antigens on ovarian carcinomas. The best documented anti-carcinoma antibodies with specificities for ovarian cancer are: HH8 (anti-Galb (1-3) GalNac-a-O-Ser/Thr) (Kjeldsen et al, 1974), MLS-128 (anti Tna = GalNac-a-O- Ser/Thr) (Nakada et al, 1992), anti CEA (Hammarstršm et al,1989), B72.3 (Johnsson et al, 1986), CA125 (Bast et al, 1987), KS-1/4 (Bumol et al, 1988) and BR 96 (anti-Le Y, Hellstršm et al,1990) stain differently in IHC and flow cytometry. The EXT1-7C12 antibody stains ovarian carcinomas to an equal or better intensity than other antibodies as judged from the literature and in direct comparing experiments on frozen tissue sections. In a serum assay for circulating antigen, the presence of EXT1-7C12 does not correlate with the most frequently used serum marker CA 125.

The antigen recognised by EXT1-7C12 seems to be a large plasma membrane associated glycoprotein differing from the well characterised MUC-1,2,3,4 and 5 mucins. EXT1-7C12 gives a much lower normal tissue binding compared to other anti-mucin antibodies. Taken together with the fact that both radiolabelled and unconjugated EXT1-7C12 antibody localised to the NIH:OVCAR-3 explanted tumours, but not to antigen negative melanoma in SCID mice, EXT1-7C12 looks like a promising antibody for diagnostic and therapeutic use in patients suffering from ovarian carcinoma.

Table 1

Ability of EXT1-7C12 to bind to cell lines

Cell line	Tumour origin	Binding
COLO 205	Colon cancer	-
PANC-1	Pancreatic cancer	-
BxPc		-
MIA PaCa-2		-
ASPC-1		-
Caov-3	Ovarian cancer	+
SK-OV-3		-
NIH:OVCAR-3		+
MCF7	Breast cancer	-
T-47		-
PC3	Bladder cancer	+
PA1	Teratoma	-
HEL8	Embryonal Lung Fibroblasts	-
A 549	Lung cancer	+
Jurkat	Leukaemia (ALL)	-

Different cell lines were stained with the EXT1-7C12 antibody in an ELISA test. Bound antibody was detected with HRPO-labelled rabbit anti-murine Ig. Wells with a light absorbance three times above the background were regarded as positive (+)

Table 2

Ability of the EXT1-7C12 antibody to bind to cell lines as analysed by flow cytometry

Cell line	Tumour origin	Per cent positive cells
COLO 205	Colon cancer	1
PANC-1	Pancreatic tumour	5
MIA PaCa-2		31
ASPC-1		5
BxPc		2
Caov-3	Ovarian cancer	13
SK-OV-3		4
NIH:OVCAR-3		99
T-47	Breast cancer	3
PC3	Bladder cancer	3
PA1	Teratoma	2
HEL8	Embryonal Lung Fibroblasts	2
A 549	Lung cancer	30

The cells were stained in suspension using a FITC-labelled secondary anti-mouse Ig antibody.

Table 3

Immunohistochemical analysis of binding of the EXT1-7C12 antibody to tissue sections.

Tissue	# positive/ # tested	Positive cells
Normal		
Brain	0/8	
Peripheral nerve	0/1	
Lung	0/9	
Liver	0/10	
Kidney	0/12	
Ovary	0/7	
Mammary gland	3/4	A few cells in ductal epithelium
Oesophagus	0/1	
Stomach	5/11	A faint staining of some mucin in the upper mucosa
Colon	10/15	A faint staining of some epithelial cells in crypts
Tonsil	0/13	
Lymphnode	0/8	
Pancreas	0/4	
Muscle	0/12	
Peyers plaque /colon	0/10	
Placenta	0/4	
Tumour		
Colo/rectal	2/14	
Ovarian	10/14	
Lung	5/15	
Pancreatic	1/8	
Breast	5/10	

Table 4

Immunohistochemical analysis of binding of the EXT1-7C12 antibody to paraffin embedded tissue sections of different ovarian tumours.

Class of ovarian tumour	Degree of differentiation	EXT1-7C12 binding
Cystadenomas, serous	Low	++
	Low	-
	Low	++
	Medium	+
	Medium	++
	Medium	+
	Medium	+
	High	+
	High	++
	High	+
Cystadenom, mucinous		-
Cystadenom, mucinous		-
Cystadenom, mucinous		+/-
Cystadenom, mucinous		-
Cystadenom, mucinous		-
Borderline tumour		++
Endometroid tumour		+++
Endometroid tumour		+/-
Endometroid tumour		-

Table 5

Immunohistochemical analysis of four monoclonal antibodies used in clinical trials.

Normal tissue	17-1A	KS1/4	HMFG-1	B72.3
Brain	0/6	6/6 Staining of blood vessels	0/6 Brain	6/7 Staining of cell nuclei and some blood vessels
Peripheral nerve	0/1	1/1 Nerve fibre	0/1	0/1
Lung	0/7	7/7 Columnar epithelium	7/7 Mucin	0/7
Liver	0/7	7/7 Bile ducts	0/7	0/7
Kidney	4/4 some tubules	4/4 Some tubules	4/4 Some tubules	3/4 Few tubules
Ovary	0/5	5/5 Connective tissue	0/5	0/5
Mammary gland	0/3	3/3 Cells in ductal epithelium	3/3 Mucins associated with cells in ductal epithelium	3/3 Some spots of mucins associated with cells in ductal epithelium
Oesophagus	1/1 epithelia	1/1 Epithelia	1/1 Mucin associated	1/1 Mucin associated
Stomach	9/8 Surface epithelium of mucosa. Parietal cells and cells in gastric glands	8/8 Surface epithelium of mucosa. Parietal cells and cells in gastric glands	9/8 Mucin of mucosa, heterogeneous staining of gastric glands	3/8 Mucin of mucosa
Colon	10/10 Epithelial cells in mucosa	10/10 All epithelial cells in mucosa.	10/10 Mucin and weak staining of all cells in crypts of mucosa. Muscle and muscularis mucosae.	10/10 Mucin associated

Table 5 (continued)

Normal tissue	17-1A	KS1/4	HMFG-1	B72.3
Tonsil	0/11	10/11 Squamous epithelial cells	10/10 Outer portion on some stratified squamous epithelial cells	10/10 Staining of the most upper layer of some mucosa
Lymphnode	0/3	1/3 Epithelial cells	0/3	0/3
Pancreas	5/6 Some acinar cells	5/6 Acinar cells	5/6 Mucin in acinar cells	4/6 Weak staining of some acinar cells
Muscle	0/10	10/10 Patched staining in different types of muscle	10/10 Diffuse staining of all muscles	0/10
Peyers plaque / colon	0/10	0/10	0/10	0/10
Placenta	1/4 Blood vessels in foetal part	4/4 Blood vessels in foetal part	3/4 Outer portion of some syncytio-trophoblasts	0/4
Tumour tissue				
Colo/rectal	15/15 Intense homogeneous staining	15/15 Intense homogeneous staining	12/15 Apical staining of cells but not stronger than to normal colon	13/15 Heterogeneous apical staining
Ovarian	8/9	9/9	4/4	6/11
Lung	20/22	6/22	ND	3/3
Pancreatic	3/3	3/3	3/3	3/3
Breast	3/6	7/7	7/7	3/6

Table 6

Binding of EXT1-7C12 and KS 1/4 monoclonal antibodies to tumour and normal tissue as assessed by immunohistochemistry

	NIH:OVCAR		A549		Kidney		Tonsilla		Ovarium		Lung	
µg/ml	EXT1 - 7C12	KS 1/4	EXT1 -7C12	KS 1/4	EXT1 -7C12	KS 1/4	EXT1- 7C12	KS 1/4	EXT1- 7C12	KS 1/4	EXT1 -7C12	KS 1/4
100	+++	++	++	++	(+)	++	(+)	-	-	+	+	++
10	+++	++	++	++	-	++	-	-	-	-	-	+
5	++	++	++	++	-	++	-	-	-	-	-	+
2.5	++	++	++	++	-	++	-	-	-	-	-	+
1.25	++	++	++	++	-	++	-	-	-	-	-	+
0.6	++	+	++	++	-	+	-	-	-	-	-	(+)
0.3	++	+	++	+	-	+	-	-	-	-	-	(+)
0.15	++	(+)	++	-	-	(+)	-	-	-	-	-	(+)
0.07	+	-	+	(-)	-	(+)	-	-	-	-	-	(+)
0.01	+	-	-	-	-	-	-	-	-	-	-	-
0.001	(+)	-	-	-	-	-	-	-	-	-	-	-
0.0001	-	-	-	-	-	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-

- to +++ designates subjective evaluation of the staining intensity

Table 7

Ability of radio labelled EXT1-7C12 antibody to bind to NIH:OVCAR-3

125 I labelled 7C12 ng	CPM bound B	CPM free F	(ng bound) B
100	167167	143301	54
50	112323	54834	36
25	43377	22301	14
12,5	20612	9640	6,8
6,25	14947	4748	5,0
3,12	8375	2164	2,9
0,78	954	231	0,5

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Uitendaal M.P., Debruyn C.H.M.M. et al.(1979). An ultramicrochemical test for mycoplasmal contamination of cultured cells. In Vitro, 15, 103-108.

Xu M., Real F.X. et al. (1989). Expression of TAG-72 in normal colon, transitiona mucosa, and colon cancer. Int. J. Cancer., 44, 985-989.

Xu M., Real F.X. et al. (1990). O-linked mucin-type glycoproteins in normal and malignant colon mucosa: lack of T antigen expression and accumulation of Tn and sialosyl-Tn antigens in carcinomas. Int. J. Cancer, 45, 666-672.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line 7 to 14

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

BELGIAN COORDINATED COLLECTION OF MICROORGANISMS (BCCM)

Address of depositary institution *(including postal code and country)*

LABORATORY OF MOLECULAR BIOLOGY
UNIVERSITY OF GENT
K.L. LEDEGANCKSTRAAT 35
B-9000 GENT
BELGIUM

Date of deposit	Accession Number
14 MARCH 1995	LMBP 1360 CB

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

The expert solution is hereby requested, that is until the date of the grant of the applications in the various designated states or until the date on which such applications are refused or deemed to be withdrawn, a sample of the above deposit shall only be made available to an independent expert.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☑ This sheet was received with the international application

Authorized officer

G C Stradbett

For International Bureau use only

☐ This sheet was received by the International Bureau on:

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CLAIMS:

1. An antibody having the substantially the same binding specificity as monoclonal antibody EXT1-7C12 produced by the hybridoma cell line deposited at BCCM under reference LMBP1360CB.

2. An antibody as obtainable from the hybridoma cell line deposited at BCCM under reference LMBP1360CB.

3. A mutant, derivative, functional equivalent or fragment of an antibody according to claim 1 or claim 2.

4. An antibody according to claim 3 wherein the antibody is humanised.

5. Hybridoma cell line deposited at BCCM under reference LMBP1360CB.

6. A pharmaceutical composition comprising an antibody according to any one of claims 1 to 4, in combination with a pharmaceutically acceptable carrier.

7. The antibodies according to any one of claims 1 to 4 for use in a method of medical treatment or diagnosis.

8. The antibodies according to claim 7 for use in cancer therapy or diagnosis.

9. The antibodies according to claim 8 wherein the cancer is ovarian cancer.

10. The antibodies according to any one of claims 7 to 9 wherein the antibodies are for use in targeting cancer cells.

11. The antibodies according to claim 10 wherein the

antibodies are conjugated to a cytotoxic compound or a label, the antibodies being capable of selectively binding to the cancer cells so that the cancer cells can be killed or detected.

12. Antibodies according to claim 11 wherein the cytotoxic compound is a radioisotope, a drug or a toxin.

13. The use of the antibodies of any one of claims 1 to 4 in the preparation of a medicament for the treatment or diagnosis of cancer.

14. A method of determining the concentration or presence of cancer cells in vitro using the antibodies of any one of claims 1 to 4, the antibodies being associated with a label, the method comprising exposing cells in a test sample to the antibodies and determining the extent of binding by detecting the label.

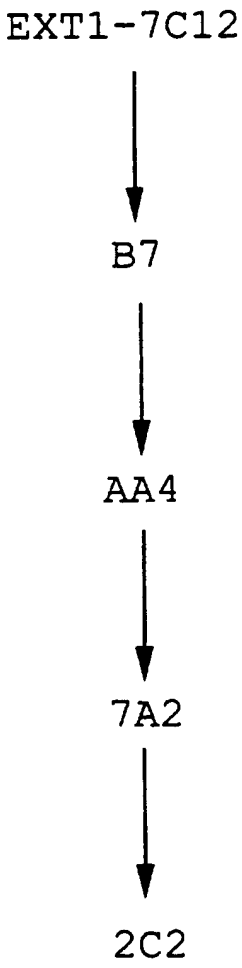


Figure 1

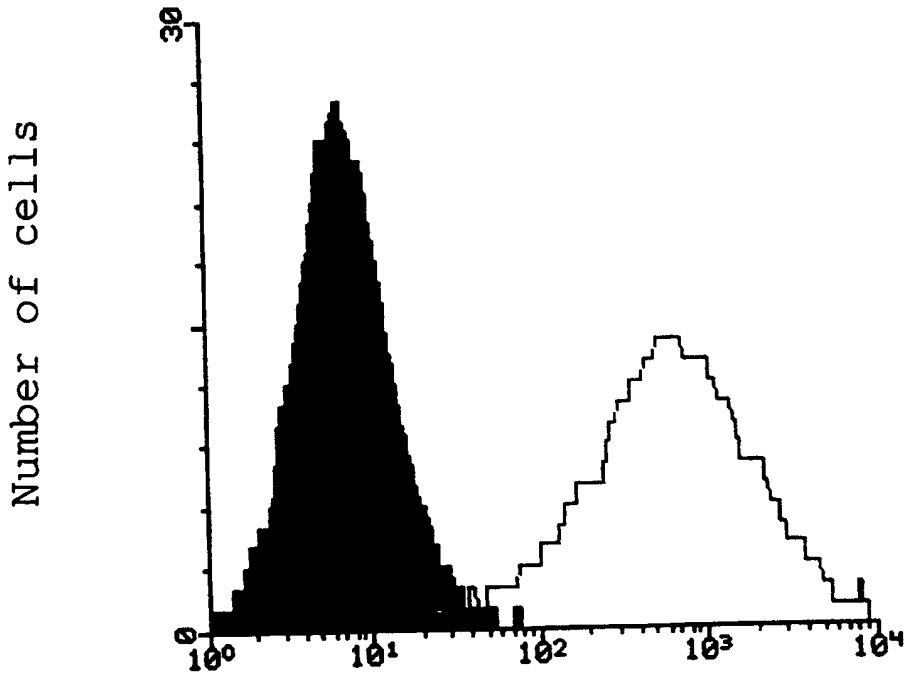

Number of cells
30
0
10^0
10^1
10^2
10^3
10^4
Log fluoresence intensity

Figure 2

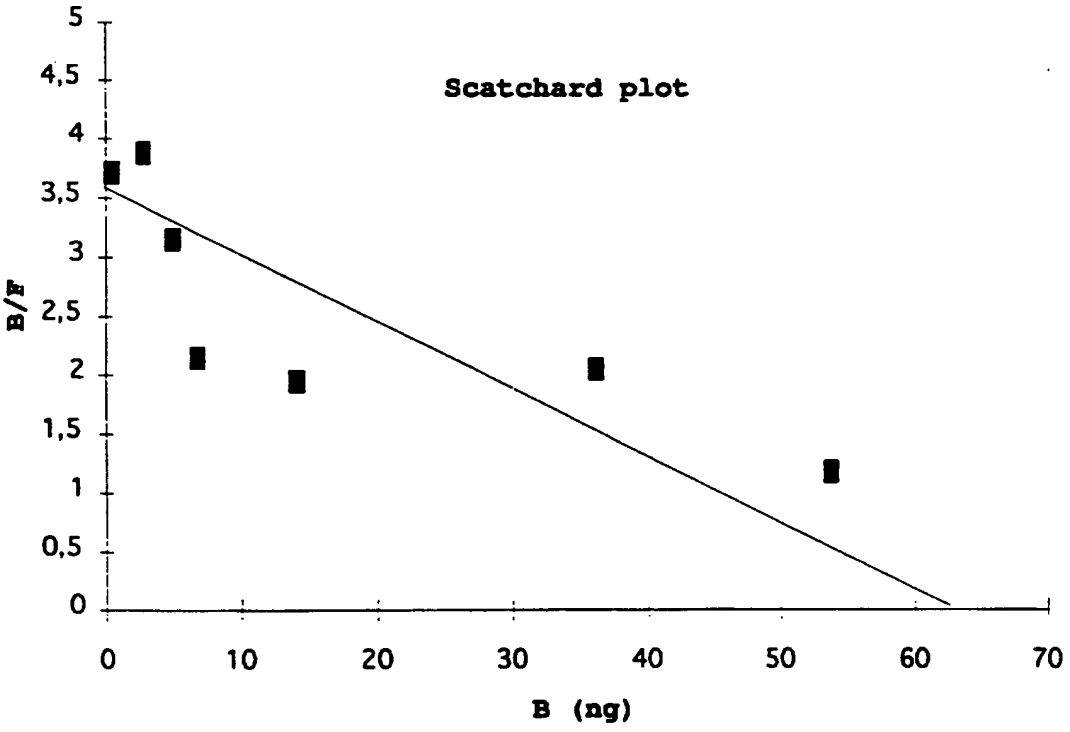


Figure 3

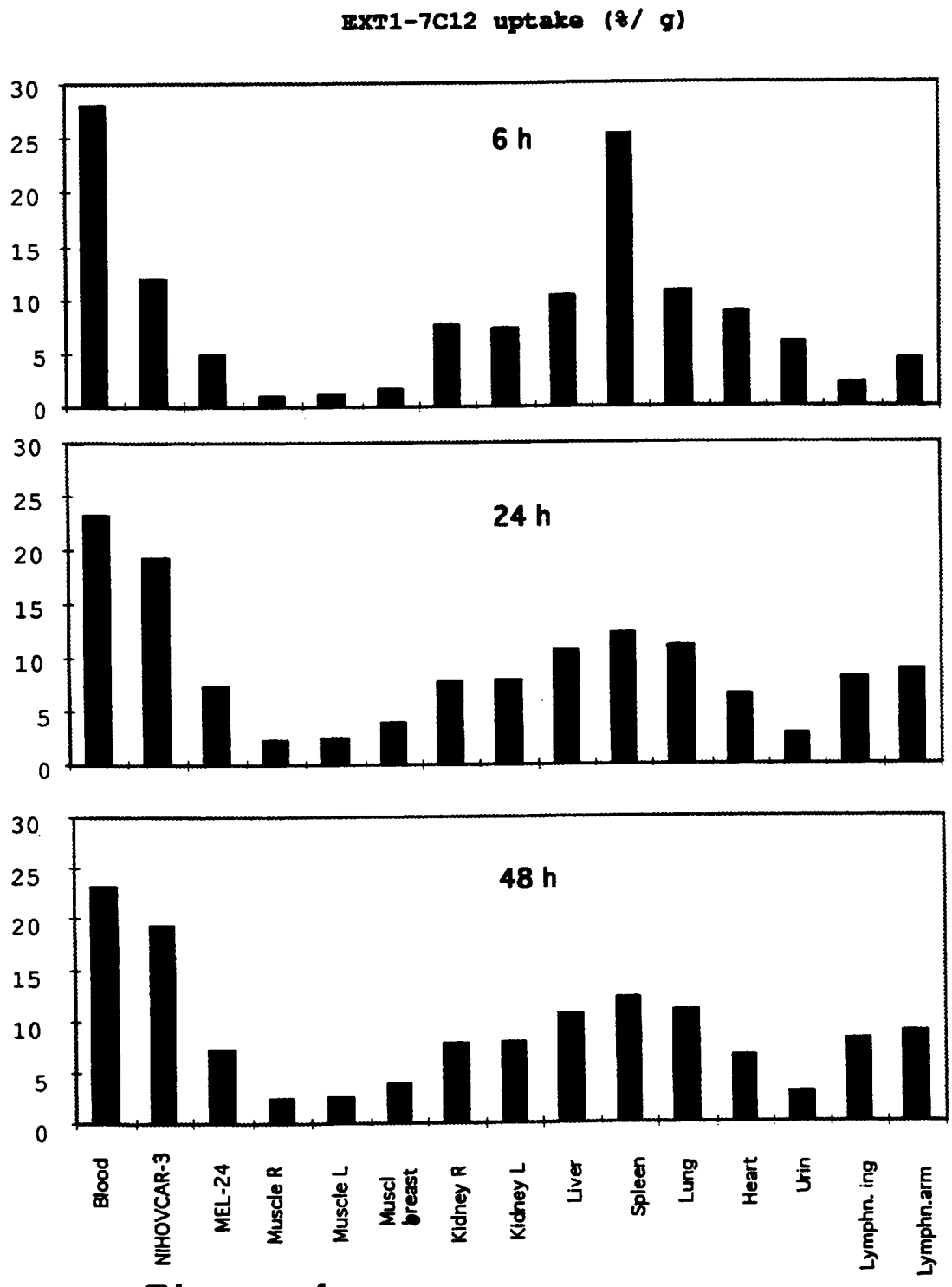

EXT1-7C12 uptake (%/ g)
6 h
24 h
48 h
30
25
20
15
10
5
0
Blood
NIHOVCAR-3
MEL-24
Muscle R
Muscle L
Muscl breast
Kidney R
Kidney L
Liver
Spleen
Lung
Heart
Urin
Lymphn. ing
Lymphn.arm

Figure 4a

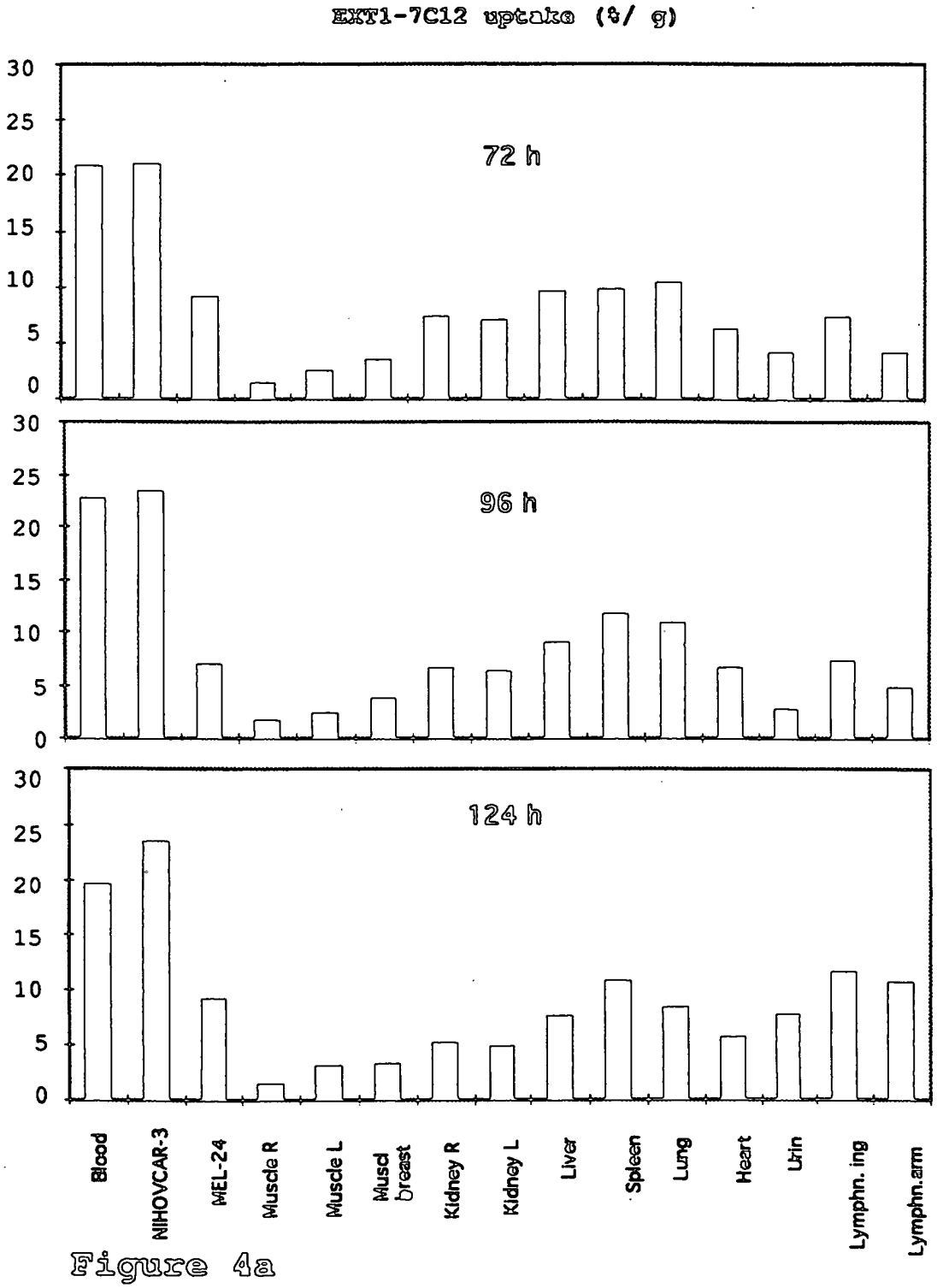

EXT1-7C12 uptake (%/ g)
72 h
96 h
124 h
30
25
20
15
10
5
0
Blood
NIHOVCAR-3
MEL-24
Muscle R
Muscle L
Musd breast
Kidney R
Kidney L
Liver
Spleen
Lung
Heart
Urin
Lymphn. ing
Lymphn.arm

Figure 4a

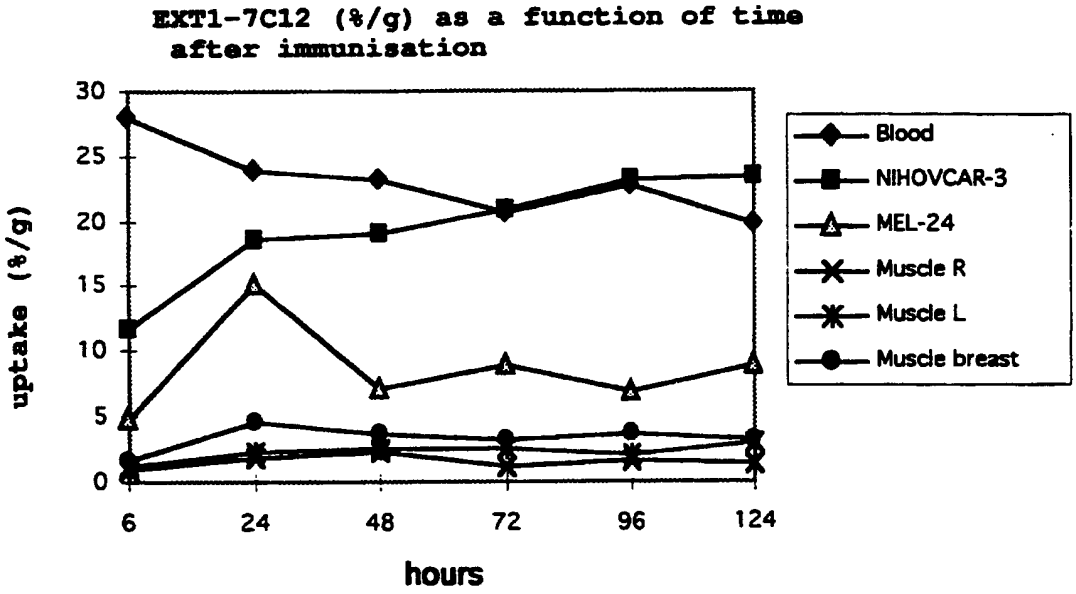

EXT1-7C12 (%/g) as a function of time after immunisation
uptake (%/g)
30
25
20
15
10
5
0
6
24
48
72
96
124
hours
Blood
NIHOVCAR-3
MEL-24
Muscle R
Muscle L
Muscle breast

Figure 4b.

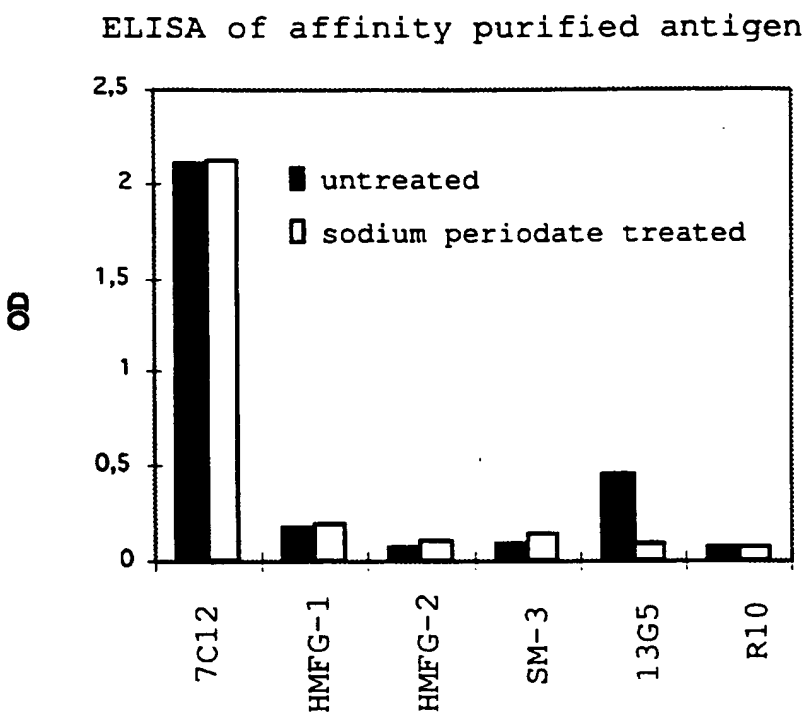

ELISA of affinity purified antigen
OD
2,5
2
1,5
1
0,5
0
untreated
sodium periodate treated
7C12
HMFG-1
HMFG-2
SM-3
13G5
R10

Figure 5a

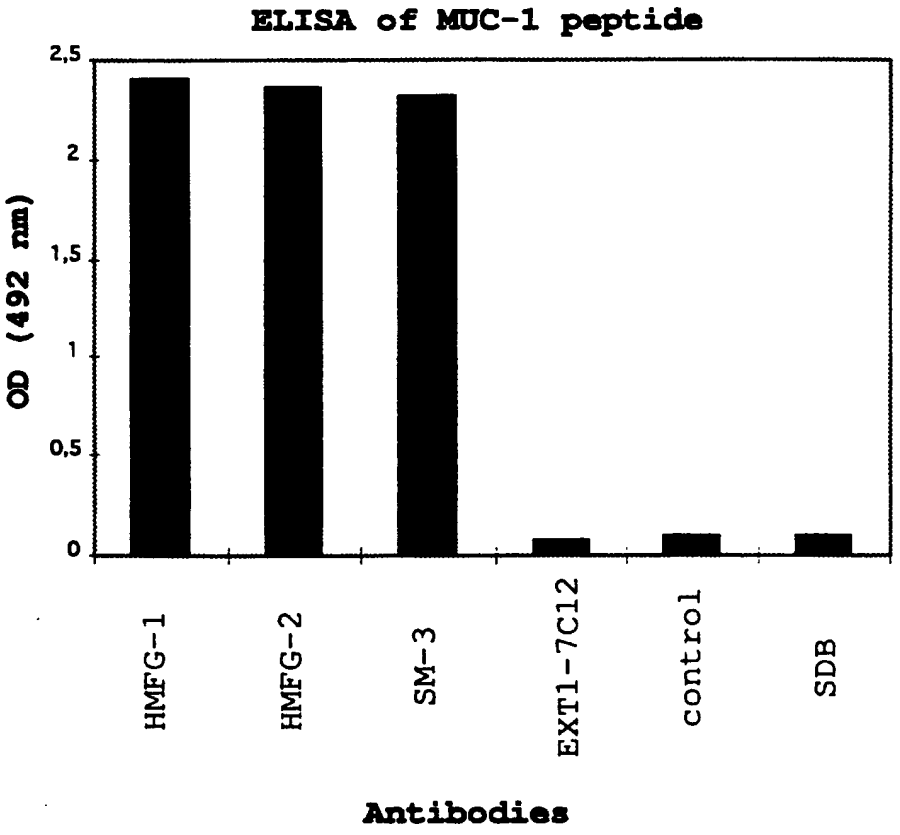
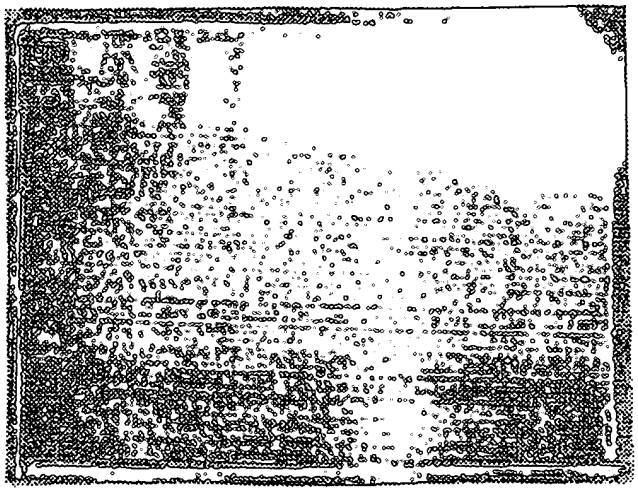

ELISA of MUC-1 peptide
OD (492 nm)
2,5
2
1,5
1
0,5
0
HMFG-1
HMFG-2
SM-3
EXT1-7C12
control
SDB
Antibodies

Figure 5b

a)



b)

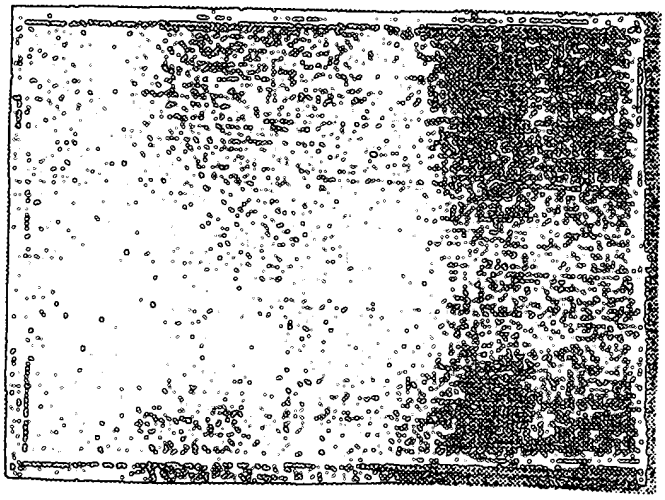


Figure 6

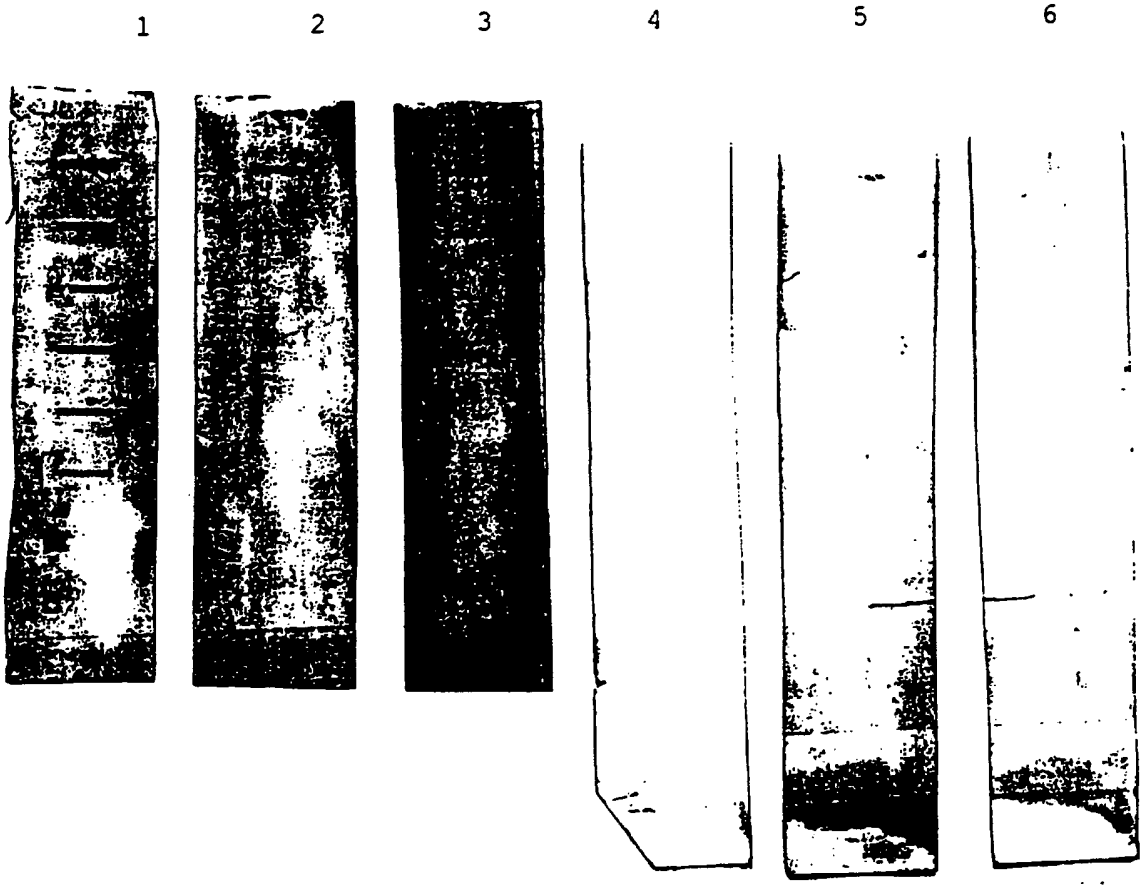


Figure 7

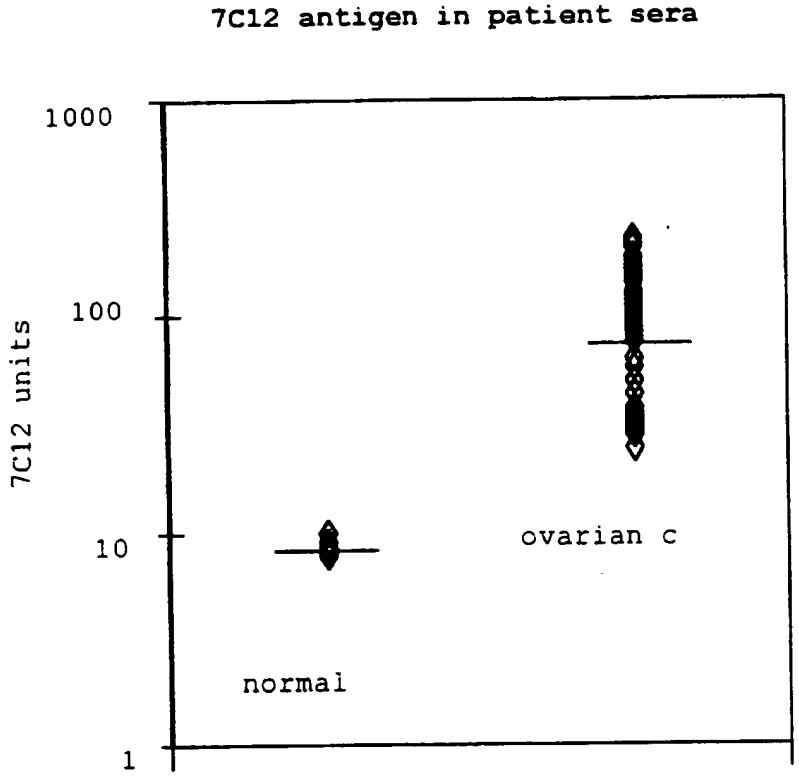

7C12 antigen in patient sera
1000
100
10
1
7C12 units
normal
ovarian c

Figure 8

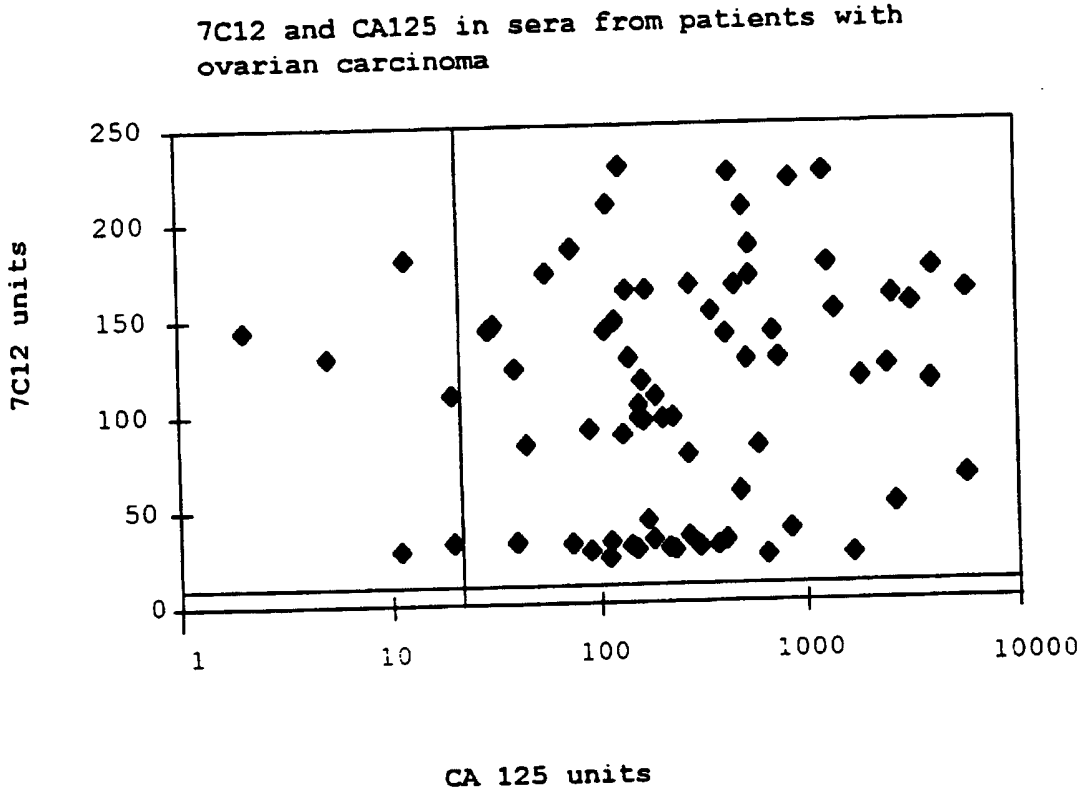

7C12 and CA125 in sera from patients with ovarian carcinoma
7C12 units
250
200
150
100
50
0
1
10
100
1000
10000
CA 125 units

Figure 9

INTERNATIONAL SEARCH REPORT

International Application No
PC1/GB 96/00783

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/30 A61K47/48 A61K49/00 A61K39/395 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 8942 Derwent Publications Ltd., London, GB; Class B04, AN 89-303484 XP002009800 & JP,A,01 211 497 (AICHI-KEN) , 24 August 1989 see abstract ---	1,3,4,14
X	WO,A,92 07081 (US ARMY) 30 April 1992 see the whole document ---	1,3,4, 6-9,13
X	PATENT ABSTRACTS OF JAPAN vol. 018, no. 351 (C-1220), 4 July 1994 & JP,A,06 090785 (AICHI PREF GOV), 5 April 1994, see abstract --- -/--	1,3,4, 7-9,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
31 July 1996	09. 08. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 226 419 (CETUS CORP ;US HEALTH (US)) 24 June 1987 see the whole document ---	10,11
A	WO,A,93 02358 (UNIV DUKE) 4 February 1993 see the whole document ---	14
A	DATABASE WPI Section Ch, Week 9416 Derwent Publications Ltd., London, GB; Class B04, AN 94-131287 XP002009801 & JP,A,06 078 787 , 22 March 1994 see abstract -----	

INTERNATIONAL SEARCH REPORT

.formation on patent family members

Interr[illegible]al Application No
PC[illegible]/GB 96/00783

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9207081	30-04-92	AU-B-	648363	21-04-94
		AU-B-	8921991	20-05-92
		CA-A-	2093928	13-04-92
		EP-A-	0554356	11-08-93
		JP-T-	6506824	04-08-94
		US-A-	5525337	11-06-96
		US-A-	5320956	14-06-94
EP-A-0226419	24-06-87	CA-A-	1289880	01-10-91
		DE-A-	3686313	10-09-92
		JP-A-	62215600	22-09-87
		US-A-	4958009	18-09-90
WO-A-9302358	04-02-93	US-A-	5366866	22-11-94
		AU-B-	661332	20-07-95
		AU-B-	2415792	23-02-93
		CA-A-	2113801	04-02-93
		EP-A-	0598000	25-05-94
		JP-T-	6509641	27-10-94
		US-A-	5486456	23-01-96